Rapid generation of multiplexed and conditional CRISPR transgenic mouse models

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Abstract

Cancer is a genetically heterogeneous disease in which altered expression of genes result in a disruption in normal somatic cell growth. Researchers have developed numerous mouse models of cancer that rapidly and accurately model the disease. This has largely relied on the generation of transgenic mice expressing the Cre recombinase system, which can be used to delete or activate specific genes in a particular cell/tissue type in the mouse. It is very laborious and time consuming to generate the Cre-based models of cancer developing, most requiring the breeding of 3-4 different transgenic models into a single mouse. This barrier has largely precluded their use for pre-clinical drug testing. To address this barrier, we plan to utilize the CRISPR/Cas9 nuclease system to delete or activate specific genes that will produce cancer in the same exact fashion as the Cre-based models. Critically, we implemented multiplexed CRISPR gRNAs that can target 10 or more genes at one time, with a single transgenic mouse. We also targeted CRISPR activity in a conditional manner, allowing us to target only the cells or tissues we desire. Using this method, we will be able to generate experimental mice from a single breeding regardless of the number of genes to be deleted or activated, removing the need for laborious and costly breeding schemes to generate mouse models of cancer based on the Cre system. We engineered this CRISPR system in mouse embryonic stem (ES) cells that will be used to generate our multiplexed conditional CRISPR (MCC) transgenic animals. Subsequent MCC mice will be bred and aged for tumor development to validate our system.

Multiplexed guide array through Golden Gate assembly

Using older techniques from TALENs and the newer technology of CRISPR/Cas9, it is possible to assemble an array of guide RNAs (gRNA) for targeting multiple genes at once with Cas9. The guide array is assembled using the Golden Gate assembly method of TALENs. The guide array contains truncated gRNAs with short regions of target complementarity for CRISPR/Cas9. This allows for the knockin and knockout of multiple genes through homologous recombination. A CMV promoter is included in the vector. In this experiment, we will provide 2 tumor suppressor genes, Rb1 and p53, flanked with 4 gRNAs each (a total of 8 gRNAs). This array will be conditionally expressed in mice by integration into a cassette vector.

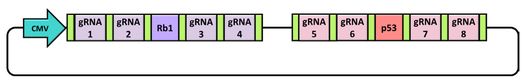

Figure 1: CMV-gRNA Array

Hypothesis

A cassette exchange system to introduce a multiplexed gRNA array into mouse embryonic stem cells will rapidly produce transgenic mouse models of cancer.

Conditional expression of guide array

A cassete vector including mCherry and dEGFP must be integrated into the Rosa26 genomic locus of the mouse ES cell. Rosa26 is a constituitive and ubiquitive locus commonly used in conditional reporter mouse lines. The Rosa26 cassette includes two FRT sites which allow for the integration of the vector containing the CMV-gRNA array previously described. The expression of the guide array will be checked by PCR and TIDE analysis. Lastly, the iCaspase 9 in the cassette acts as a kill-switch with non-integrated cells through the use of a suicide gene system with the small-molecule drug AP1903.

Figure 2: Rosa26 cassette exchange with CMV-gRNA Array using iCaspase9 as a selection for successful integration

Materials & Methods

- **Cell culture:** ES cells were co-cultured on top of a monolayer of mouse embryonic fibroblasts (MEFs). ES cells were cultured at 37 degrees C in 5% CO_2 in IMDM (20% ES cell-qualified FBS, 2mM L-glutamine, 1X non-essential amino acids, and 0.1 mM B-mercaptoethanol.)
- **Cell Transfection:** The Neon Transfection System (Thermo Fisher Scientific) was used to electroporate 1 x 10^6 million MEF-depleted ES cells with 1,400V pulse, 10ms width, 3 pulses. After electroporation, cells were placed into a fresh well of feeder cells on a 24-well plate.
- **Plasmid and vector construction:** Plasmids were constructed using BP and LR clonase enzymes.
- **Flow cytometry:** ES cells were MEF-depleted and not fixed. Cells were stained with Sytox Blue. They were run using a WT ES cell control on a BD LSRFortessa machine. All results were analysed using FlowJo.
- **Sequencing**: All sequencing was done by the UMN Genomics Center.

Integration of Rosa26 cassette

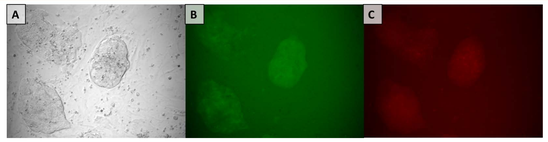

Figure 3: Mouse embryonic stem cells express GFP and mCherry after transfection with Rosa26 cassette

ES cells transfected with the Rosa26 cassette were B. GFP and C. mCherry positive. This confirms the integration of the cassette as cells express markers inside and outside of FRT sites.

Cre excision of mCherry

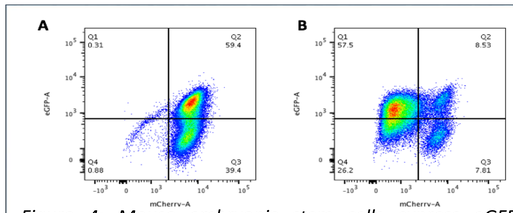

Figure 4: Mouse embryonic stem cells express eGFP and mCherry before and after Cre excision.

Flow cytometry assay comparing the expression of eGFP and mCherry. A. Mouse embryonic stem cells with no Cre excision enables a double positive expression of eGFP and mCherry
B. Cells with Cre excision are eGFP positive and have reduced mCherry expression.

BFP/Puro/TK plasmid construction

Once cells had a confirmed integration of the Rosa26 cassette, the iCaspase9 kill switch selection marker was tested by adding the small molecule drug AP1903 to the cells. After no change was observed after drug addition, it was concluded that a new selection marker must be employed through a new cassette exchange by FRT sites with a BFP/Puro/TK plasmid. This plasmid was constructed with a PGK promoter.

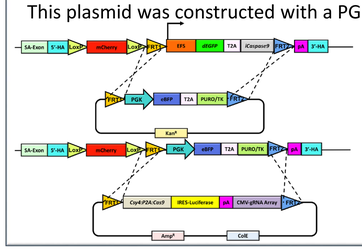

Figure 5: Cassette exchange from Rosa26 Cassette to BFP/Puro/TK cassette. Subsequent exchange with the CMV gRNA array is illustrated

BFP Puro-TK plasmid expression in 293Ts

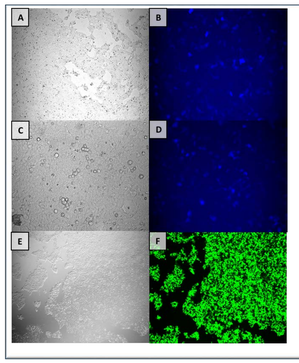

Figure 6: Transient expression of the BFP Puro-TK plasmid in HEK293T cells under bright field and BFP fluorescence to test for correct construction of plasmid.

A/B. 2ug of BFP Puro-TK plasmid. C/D: 6ug of BFP Puro-TK plasmid E/F: 1ug of GFP pmax control

Summary

- Mouse ES cells can integrate the Rosa26 Cassette
- Correct integration was confirmed using Cre/LoxP excision of mCherry and sequencing
- Selection of ES cells using iCaspase9 does not work
- BFP-PuroTK plasmid constructed for cassette exchange

Future Directions

- Insert the BFP Puro-TK into the cassette in mouse ES cells using Flp plasmid
- Confirm successful integration of BFP Puro-TK for further use as a selective marker
- Exchange the CMV-gRNA array with the BFP/PuroTK cassette and test for knockdown of Rb1 and p53 genes
- Implant cells into pseudo-pregnant mice and breed/age for cancer development

Acknowledgements

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